Quality of raw and of cold-stored semen in Icelandic stallions

Janett, F ; Sacher, K ; Hässig, M ; Thun, R

Abstract: The aim of the present study was to evaluate the quality of raw and cooled semen in Icelandic stallions. Experiments were performed using seven stallions aged between 3 and 19 years. From each stallion, six ejaculates were collected, and semen quality was determined. Thereafter, the semen was split into eight equal parts and processed with and without centrifugation using the extenders INRA 82-egg yolk, INRA 96, GENT, and Equi-Pro to a final concentration of 30 × 106 sperm/mL. The extended semen was then cooled in an Equitainer, where it was stored for 24 hours, and subsequently refrigerated for another 24 hours at 5°C. Immediately after dilution as well as after 24 and 48 hours storage, sperm motility was analyzed using computer-assisted sperm analyzer, and viability was assessed after dual DNA staining with SYBR-14 in combination with propidium iodide. The results show that the stallion had a significant ($P < .05$) influence on all variables evaluated in raw semen, and mean (±SEM) values of 43.4 ± 4.3 mL for the volume, 193.0 ± 17.0 × 106 sperm/mL for the concentration, 6.7 ± 0.5 × 109 for total sperm and 73.5 ± 2.1% for total sperm motility, 48.7 ± 2.0% for progressive motility, and 65.3 ± 2.0% for rapid cells were measured. In the cold-stored semen, all variables were significantly ($P < .05$) influenced by the stallion, extender, and storage time (48 hours). Except for Equi-Pro, all extenders examined were suitable for cooled semen preservation. For storage of more than 24 hours, centrifugation and removal of the seminal plasma were advantageous for all extenders with the exception of Equi-Pro.

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Fredi Janett PD Dr. med. vet.[a], Konrad Sacher Dr. med. vet.[a], Michael Hassig Prof. Dr. med. vet.[b], Rico Thun Prof. Dr. med. vet.[a]

[a] *Clinic of Reproductive Medicine, Vetsuisse-Faculty University of Zürich, Switzerland*

[b] *Department of Farm Animals, Vetsuisse-Faculty University of Zürich, Switzerland*

Correspondence author at: Fredi Janett PD Dr. med. vet., Clinic of Reproductive Medicine, Vetsuisse-Faculty University of Zürich, Winterthurerstrasse 260, CH-8057 Zürich

E-mail address: fjanett@vetclinics.uzh.ch (F. Janett)

Abstract

The aim of the present study was to evaluate the quality of raw and cooled semen in Icelandic stallions. Experiments were performed using 7 stallions aged between 3 and 19 years. From each stallion 6 ejaculates were collected and semen quality determined. Thereafter, the semen was split into 8 equal parts and processed with and without centrifugation using the extenders INRA 82-egg yolk, INRA 96™, GENT™ and Equi-Pro™ to a final concentration of 30 x 10^6 sperm/mL. The extended semen was then cooled in an Equitainer™, where it was stored for 24 h, and subsequently refrigerated for another 24 h at 5 °C. Immediately after dilution as well as after 24 and 48 h storage, sperm motility was analyzed using CASA and viability assessed after dual DNA staining with SYBR-14 in combination with propidium iodide. The results show that the stallion had a significant ($P < 0.05$) influence on all variables evaluated in raw semen and mean (±SEM) values of 43.4±4.3 mL for the volume, 193.0±17.0 x 10^6 sperm/mL for the concentration, 6.7±0.5 x 10^9 for total sperm and 73.5±2.1% for total sperm motility, 48.7±2.0% for progressive motility and 65.3±2.0% for rapid cells were measured. In the cold-stored semen, all variables were significantly ($P<0.05$) influenced by the stallion, extender and storage time (48 h). Except for Equi-Pro™, all extenders examined were suitable

for cooled semen preservation. For storage of more than 24 h, centrifugation and removal of the seminal plasma were advantageous for all extenders with the exception of Equi-Pro™.

Keywords:

CASA

Extender

Icelandic horse

Semen

Stallion

1. Introduction

While artificial insemination (AI) is widely used in various horse breeds, tradition and the mainly pasture based style of horse keeping in Iceland dictates that by far the most foals are born out of matings between a stallion running with a herd of mares [1,2]. Although a reproductive unit was established in Holar in Iceland to promote AI and embryo transfer, AI has failed to make a big impact as yet and operations were not continued. However, with the sustained popularity of the horse breed throughout Europe and the growing demand for the services of particularly successful stallions, the demand for AI with raw, chilled and frozen semen is gradually increasing. Presently, there is very little information about the success rates of AI in Icelandic horses and there are no reports about semen quality in Icelandic stallions or of the suitability for semen preservation.

Thus, the aim of this study was to evaluate raw semen characteristics in Icelandic stallions and the influence of 4 proven extenders on the quality of cold-stored semen.

2. Materials and Methods

2.1. Experimental design

The experiment was carried out between April and October using 7 healthy Icelandic stallions aged between 3 and 19 years. For the duration of the experiment, the animals were kept in individual boxes bedded with straw and exercised daily. They were fed hay, oats and pellets supplemented with minerals. Before beginning the experiment, the extragonadal sperm reserves were minimized in each stallion by daily semen collections over a period of 5 consecutive days. Thereafter, 6 ejaculates were collected from each stallion over 3 consecutive weeks with 2 semen collections per week (Monday and Wednesday). Semen collection was performed on a phantom (Dummy Mare, Arnolds™, Shrewsbury, Great Britain) using a Hannover model artificial vagina (Minitüb, Landshut, Germany) in the presence of a mare.

2.2. Semen examination and processing

Immediately after semen collection the gel fraction was removed via in-line gel filter, the volume of the gel-free ejaculate estimated and the semen placed in a water bath at 37 °C. Sperm concentration, motility and total sperm count of the ejaculate were assessed with a computer assisted sperm analyzer (CASA) (Hamilton Thorne IVOS, Version 14, Beverly, MA, USA) using standardized settings for stallion semen (Table 1).

Table 1

CASA settings for stallion semen.

Parameter	Setting
Frames aquired	30
Frame rate	60 Hz
Minimum contrast	115
Minimum cell size	2 pix
Minimum static contrast	15
Straightness (STR), threshold	50 %
VAP cutoff	5.0 µm/s
Prog. min. VAP	50.0 µm/s
VSL cutoff	15.0 µm/s
Cell size	6 pix
Cell intensity	125
Static head size	0.70 to 3.60
Static head intensity	0.25 to 2.10
Static elongation	0 to 100
Slow cells motile	NO
Magnification	1.89
Video frequency	60
Bright field	NO
LED illumination intensity	2280
IDENT Illumination intensity	3000
Temperature, set	37.5 °C
Chamber depth	20 µm
Chamber position	4.0 mm
Chamber position B	19.0 mm
Chamber position C	0 mm
Chamber position D	0 mm
Chamber type	Slide20
Field selection mode	AUTO
IDENT fluorescent option	OFF
Integrating time	1 frame

For the measurements 10 µL raw semen was diluted with 20 µL INRA 96^{TM} (IMV Technologies, Saint Ouen Sur Iton, France). Five µL of the diluted semen were pipetted into a 20 µm standard count

analysis chamber (Art. Nr. SC 20-01-C, Leja, Nieuw-Vennep, Netherlands) and a minimum of 10 fields evaluated by CASA. The average percentage of total and progressive motility as well as of rapid sperm cells (velocity average path VAP> 50 µm/s) were determined. To assess the quality of chilled stored semen and the suitability of various extenders, the ejaculates were divided into 8 equal parts, placed in 15 mL centrifuge tubes and diluted with the extenders INRA 82-egg yolk, INRA 96™, GENT™ and Equi-Pro™ to a final concentration of 30 x 10^6 sperm/mL (two tubes per extender).

INRA 82 was prepared according to Palmer [3] and supplemented with 2% centrifuged egg yolk.

INRA 96™ is patented and available as a ready-to-use solution (REF. 016441, IMV Technologies, Saint Ouen Sur Iton, France). This chemically defined extender contains Hank's salts, glucose, lactose and native phosphocaseinate [4].

GENT™-extender was developed at the University of Ghent (Belgium) and consists of buffers, milk and egg yolk and is marketed by Minitüb (REF. 13571/0045, Minitüb, Tiefenbach, Germany).

Equi-Pro™ (REF. 13570/0210, Minitüb, Tiefenbach, Germany) is based on the the Kenney extender [5] and contains defined caseinates, whey proteins, glucose, sucrose and an antibiotic (gentamicin sulfate). It was used in the powdered form and dissolved in purified water (bidistilled, sterilized, pyrogen-free water) according to the manufacturer's instructions.

After dilution, aliquots of 10 mL of each of the 8 portions of extended ejaculate were placed in 15 mL centrifuge tubes, and 4 of the samples (one of each extender) were processed by centrifugation. Centrifugation was performed at 600 x g for 10 min at room temperature [6], thereafter 90% of the supernatant were removed and the sperm pellet resuspended in the corresponding extender to a final concentration of 30 x 10^6 sperm/mL. After centrifugation both the centrifuged and not centrifuged semen tubes were placed in an Equitainer™ (Hamilton Thorne Beverly, MA, USA) and cooled to approximately 5 °C for 24 h [7]. Thereafter, the semen was placed in a refrigerator and stored for further 24 h at approximately 5 °C.

In all centrifuged and not centrifuged semen samples, sperm motilty and viability were evaluated immediately after final dilution, after 24 h of storage in the Equitainer™ and after additional 24 h storage in the refrigerator. Sperm motility was assessed by CASA as in raw semen evaluation but

without further dilution. The percentage of total and progressive motility as well as of rapid sperm cells (velocity average path VAP>50 µm/s) were considered. Determination of sperm viability was performed by dual DNA staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes Europe, Leiden, NL) using SYBR-14 in combination with propidium iodide (PI) [8]. SYBR-14 (component A) was diluted with anhydrous dimethyl sulfoxide (DMSO) 1:10 (SYBR-14 working solution), while PI (component B) was used in original concentration. Two µL of the SYBR-14 working solution were added to 1 mL of diluted semen. After an incubation period of 10 min at 37 °C, 5 µL of component B were added. Five min later, 5 µL of stained semen were placed on a slide, covered with a coverslip (24 x 24 mm) and examined using fluorescence microscopy (Olympus BX50, UPlanApo 40x/0.85, FITC filter U-MWIB, high pressure Hg-lamp). Different fields with fluorescence stained sperm were recorded using a video camera connected to the microscope (SANYO VCC-2972). The images were stored on a computer and analyzed using the program Windows Media Player (http://www.microsoft.com). At least 500 sperm cells were evaluated and the percentage of green fluorescing sperm was defined as viability [8].

2.3. Statistical analysis

Data were analyzed using a SPSS 13 software program (SPSS Inc., IBM Company Headquarters, Chicago, Illinois 60606, USA). The effects of stallion and ejaculate on raw semen characteristics were assessed by ANOVA with repeated measurements. Sperm motility and viability in the stored semen were analyzed using a general linear model testing the effects of stallion, extender, centrifugation and storage time as well as interactions between the different factors on variables. Post-hoc comparisons were made after Bonferroni adjustment. Statistical significance was set at $P<0.05$.

3. Results

3.1. Raw semen characteristics

The results demonstrate that the stallion but not the ejaculate significantly ($P<0.05$) influenced all parameters measured in raw semen (Table 2).

Table 2

Effects (*P* value) of stallion and ejaculate on fresh semen characteristics.

	Sperm parameter					
	Volume	Concentration	Total sperm	Total motility	Progressive motility	Rapid sperm cells
Stallion	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Ejaculate	0.7250	0.5573	0.7936	0.4280	0.1721	0.5180

*significant (*P*< 0.05)

Means (±SEM) of raw semen characteristics of individual stallions are summarized in Table 3. The average volume ranged from 21.5±2.9 mL (Stallion 5) to 94.2±10.7 mL (Stallion 7). Significant differences were present between Stallion 7 and all other stallions as well as between the Stallions 1 and 5. Mean (±SEM) sperm concentration ranged from 76.3±9.0 x 10^6 sperm/mL (Stallion 1) to 326.9±42.5 x 10^6 sperm/mL (Stallion 5). Significant differences were identified between Stallion 1 and Stallions 2, 3 and 5, between Stallion 5 and Stallions 6 and 7 as well as between Stallions 2 and 7. Mean (±SEM) total sperm count ranged from 3.8±0.5 x 10^9 (Stallion 1) to 10.2±1.3 x 10^9 (Stallion 7). Significant differences were apparent between Stallion 1 and Stallions 2 and 7 as well as between Stallion 3 and 7. Mean (±SEM) total motility ranged between 49.2±2.4% (Stallion 1) and 86.0±3.0% (Stallion 2). Significant differences were found between Stallion 1 and all other stallions as well as between Stallion 5 and Stallions 2 and 3. Mean (±SEM) progressive motility ranged from 29.3±2.5% (Stallion 1) to 67.7±2.2% (Stallion 2). Significant differences were obvious between Stallion 1 and all other stallions with exception of stallion 5, between Stallion 2 and all other stallions excluding Stallion 3 as well as between Stallion 5 and the Stallions 3 and 7. Mean (±SEM) values of rapid sperm cells varied between 44.2±2.2% (Stallion 1) and 79.3±2.5% (Stallion 2). Significant differences were present between Stallion 1 and all other stallions except Stallion 5, between Stallion 5 and all other stallions excluding Stallions 1 and 4 as well as between the Stallions 2 and 4.

Table 3

Means (±SEM) of fresh semen characteristics in 7 Icelandic stallions (n= 6 ejaculates each).

Stallion	Sperm parameter					
	Volume (mL)	Concentration ($x10^6$/mL)	Total sperm ($x10^9$)	Total motility (%)	Progressive motility (%)	Rapid cells (%)
1	[a] 52.5± 7.4	[a]76.3± 9.0	[a]3.8±0.5	[a]49.2±2.4	[a]29.3±2.5	[a]44.2±2.2
2	[ab] 35.0± 3.7	[bc]263.6±53.5	[bc]8.6±1.4	[b]86.0±3.0	[b]67.7±2.2	[b]79.3±2.5
3	[ab] 23.8± 2.2	[bcd]238.8±32.3	[ab]5.4±0.4	[b]84.0±1.8	[bd]56.8±1.8	[bd]72.7±1.5
4	[ab] 31.7± 3.6	[abcd]203.3±27.9	[abc]6.2±0.9	[c]76.7±1.8	[cd]48.8±2.0	[cd]65.0±2.1
5	[b] 21.5± 2.9	[c]326.7±42.5	[abc]6.6±0.5	[c]67.5±5.9	[ac]37.5±4.5	[ac]54.8±5.3
6	[ab] 45.3± 9.9	[abd]129.0± 8.5	[abc]5.8±1.3	[bc]79.5±2.4	[cd]49.0±2.3	[bd]73.2±2.5
7	[c] 94.2±10.7	[ad]113.1±13.3	[c]10.2±1.3	[bc]72.0±1.5	[d]51.8±2.1	[bd]68.2±1.0
Overall mean	43.4± 4.3	193.0±17.0	6.7±0.5	73.5±2.1	48.7±2.0	65.3±2.0

Different letters[abcd] within a column indicate significant ($P < 0.05$) differences between stallions.

3.2. Quality of stored semen

The effects of stallion, extender, centrifugation and storage time as well as the interactions between the different factors are shown in Table 4. It is evident that stallion, extender and storage time significantly influenced all variables. Centrifugation had a significant effect on progressive motility and on viability. For all variables, significant interaction effects were found for stallion x extender, stallion x storage time, extender x centrifugation, stallion x extender x centrifugation as well as for stallion x extender x centrifugation x storage time. The interaction stallion x centrifugation was significant in all variables with the exception of viability.

Table 4

Effect (*P* value) of stallion, extender, centrifugation and storage time as well as interactions between the different factors on the quality of cold-stored semen (n=7 Icelandic stallions, n=6 ejaculates each).

	Total motility	Progressive motility	Rapid cells	Viability
Stallion	<0.001*	<0.001*	<0.001*	<0.001*
Extender	<0.001*	<0.001*	<0.001*	<0.001*
Centrifugation	0.624	0.002*	0.342	<0.001*
Storage time	<0.001*	<0.001*	<0.001*	<0.001*
Interaction stallion x extender	<0.001*	<0.001*	<0.001*	<0.001*
Interaction stallion x centrifugation	0.004*	<0.001*	<0.001*	0.246
Interaction stallion x storage time	<0.001*	<0.001*	<0.001*	<0.001*
Interaction extender x centrifugation	<0.001*	<0.001*	<0.001*	0.001*
Interaction stallion x extender x centrifugation	<0.001*	<0.001*	<0.001*	<0.001*
Interaction stallion x extender x centrifugation x storage time	<0.001*	<0.001*	<0.001*	<0.001*

*significant ($P < 0.05$)

Means (±SEM) of sperm motility parameters assessed by CASA and sperm viability of stored (0, 24 and 48 h) semen processed with Equi-Pro™, GENT™, INRA 82-egg yolk and INRA 96™, with and without centrifugation, are shown in Table 5.

Table 5

Means (±SEM) of sperm motility parameters and sperm viability of cold-stored semen diluted with 4 different extenders and processed with and without centrifugation (n=7 Icelandic stallions, n=6 ejaculates each).

	Equi-Pro™		**GENT™**		**INRA 82-egg yolk**		**INRA 96™**	
	not centrifuged	centrifuged	not centrifuged	centrifuged	not centrifuged	centrifuged	not centrifuged	centrifuged
Total motility (%)								
- after dilution	^{a}67.3±1.7	^{b}57.7±1.8	^{a}67.4±2.0	^{a}68.0±2.0	^{a}67.0±1.9	^{ab}64.9±2.0	^{a}67.6±2.1	^{a}66.9±2.1
- after 24 h	^{b}40.9±2.5	^{b}35.3±3.1	^{a}56.1±1.9	^{a}59.5±1.9	^{a}55.7±2.1	^{a}56.9±2.0	^{a}53.6±2.9	^{a}59.0±2.2
- after 48 h	^{bc}31.1±2.5	^{b}22.0±3.4	^{ac}42.5±2.2	^{ac}50.0±2.2	^{ac}44.7±2.1	^{ac}50.5±2.3	^{c}41.0±2.9	^{a}52.8±2.5
Progressive motility (%)								
- after dilution	^{ab}39.8±1.8	^{b}32.8±1.7	^{a}42.8±2.0	^{a}42.7±2.2	^{a}42.8±1.9	^{ab}40.4±2.1	^{a}46.0±2.1	^{a}45.8±2.1
- after 24 h	^{b}23.0±1.9	^{b}20.8±2.3	^{a}34.9±1.6	^{a}38.1±1.8	^{a}32.8±1.7	^{a}36.7±1.8	^{a}32.4±2.3	^{a}37.9±1.9
- after 48 h	^{bd}15.5±1.9	^{d}12.2±2.2	^{bc}22.9±1.7	^{ac}30.4±1.9	^{c}24.6±1.6	^{ac}31.1±2.1	^{bc}22.4±2.0	^{a}33.8±2.1
Rapid cells (%)								
- after dilution	^{a}60.0±1.8	^{b}49.5±1.9	^{a}60.8±2.0	^{a}61.5±2.1	^{a}60.5±1.9	^{ab}58.2±2.0	^{a}61.8±2.1	^{a}59.4±2.2
- after 24 h	^{b}34.2±2.4	^{b}29.2±2.9	^{a}48.7±1.8	^{a}53.2±1.9	^{a}47.7±2.1	^{a}50.7±2.0	^{a}46.0±2.7	^{a}49.8±2.1
- after 48 h	^{bc}24.9±2.3	^{b}17.8±3.0	^{ac}35.2±2.1	^{ac}43.8±2.1	^{ac}37.9±1.9	^{a}44.9±2.2	^{c}33.9±2.7	^{a}45.6±2.4
Viability (%)								
- after dilution	^{ab}70.3±2.0	^{b}68.9±2.0	^{ab}76.9±1.9	^{ab}77.0±1.7	^{ab}76.5±1.5	^{a}78.3±1.7	^{ab}73.7±1.8	^{ab}76.3±1.9
- after 24 h	^{b}53.9±3.0	^{bc}60.0±2.1	^{a}71.3±1.9	^{a}71.7±1.8	^{a}69.8±1.9	^{a}72.0±1.8	^{ab}63.1±2.3	^{ac}68.5±2.1
- after 48 h	^{b}46.3±2.8	^{bc}52.6±2.3	^{a}64.8±1.8	^{a}64.0±2.2	^{a}65.7±2.1	^{a}65.0±2.2	^{ab}56.5±2.7	^{ac}61.8±2.3

Different letters^{abc} within a row indicate significant ($P < 0.05$) differences between methods.

Immediately after final dilution, the highest total motility was measured in GENT™ centrifuged. Significant differences were seen between Equi-Pro™ centrifuged and all other methods with exception of INRA 82-egg yolk centrifuged. After a storage time of 24 h, the highest total motility was measured in GENT™ centrifuged. Significant differences were found between Equi-Pro™ both centrifuged and not centrifuged and all other methods. After 48 h of storage, the highest total motility was measured in INRA 96™ centrifuged. Significant differences were found between Equi-Pro™ centrifuged and all other methods except Equi-Pro™ not centrifuged. INRA 96™ centrifuged was significantly different from INRA 96™ not centrifuged and from Equi-Pro™ both, centrifuged and not centrifuged.

Progressive motility after final dilution was highest in INRA 96™ not centrifuged. Significant differences were identified between Equi-Pro™ centrifuged and INRA 82-egg yolk not centrifuged as well as GENT™ and INRA 96™, both centrifuged and not centrifuged. After 24 h storage, progressive motility was highest in GENT™ centrifuged. Semen processed with Equi-Pro™, both centrifuged and not centrifuged, differed significantly from all other methods. After 48 h storage, progressive motility was highest in INRA 96™ centrifuged. Significant differences were apparent between Equi-Pro™ centrifuged and all other methods except Equi-Pro™ not centrifuged. Furthermore, progressive motility in INRA 96™ centrifuged differed significantly from all methods without centrifugation.

The percentage of rapid sperm cells after final dilution was highest in INRA 96™ not centrifuged. Significant differences were present between Equi-Pro™ centrifuged and all other methods with the exception of INRA 82-egg yolk centrifuged. After 24 h storage, the percentage of rapid sperm cells was highest in GENT™ centrifuged. Semen extended with Equi-Pro™, both centrifuged and not centrifuged differed significantly from all other methods. After 48 h storage, values for rapid sperm cells were highest in INRA 96™ centrifuged. Significant differences were identified between Equi-Pro™ centrifuged and all other methods except Equi-Pro™ not centrifuged. INRA 96™ not centrifuged was also significantly different from INRA 96™ and INRA 82-egg yolk, both centrifuged.

Sperm viability after final dilution was highest in INRA 82-egg yolk centrifuged. A significant difference was present between Equi-Pro™ and INRA 82-egg yolk, both centrifuged.

After 24 h storage viability was highest in INRA 82-egg yolk centrifuged. Significant differences were found between Equi-Pro™ not centrifuged and all other methods, except Equi-Pro™ centrifuged and INRA 96™ not centrifuged. Moreover Equi-Pro™ centrifuged differed significantly from GENT™ and INRA 82-egg yolk, both centrifuged and not centrifuged.

After 48 h storage viability was highest in INRA 82-egg yolk not centrifuged. Equi-Pro™ not centrifuged differed significantly from all other methods with the exception of Equi-Pro™ centrifuged and INRA 96™ not centrifuged. Significant differences were also apparent between Equi-Pro™ centrifuged and GENT™ as well as INRA 82-egg yolk, both centrifuged and not centrifuged.

4. Discussion

As yet, there is no available literature reporting on the quality and preservation of semen in Icelandic stallions. The present study, which was conducted with a total of 42 ejaculates obtained from 7 stallions, provides a detailed description of raw and cold-stored semen characteristics in the Icelandic horse. Results show that the stallion significantly influenced all parameters evaluated in raw and cold-stored semen. The large individual differences observed in semen quality correspond with findings in other horse breeds [9,10]. For the Icelandic stallions used in this study, values for mean ejaculate volume, sperm concentration, total sperm count and sperm motility were 43.4 mL, 193.0 x 10^6/mL, 6.7 x 10^9 and 73.5%, respectively. In one study [9] performed with 168 stallions of 9 different breeds, the mean values for volume, concentration, total sperm count and sperm motility were 33.7 mL, 164.1 x 10^6/mL, 6.3 x 10^9 and 76.4%. However, it is difficult to directly compare the results of different studies with varying study groups as it is known that breed [9,10], age [9], season of semen collection [10,11] as well as duration of sexual rest [11,12] may influence semen quality. To test the suitability of Icelandic stallions for the production of chilled semen, ejaculates were processed with 4 different extenders with and without removal of seminal plasma by centrifugation. Semen quality was then tested after the aliquots were stored at 5 °C for 0, 24 and 48 h. It was found that after 24 h storage,

semen extended with INRA 82-egg yolk, INRA 96™ and GENT™ showed only a 10% decrease in motility. When using Equi-Pro™ a reduction of more than 20% was evident. After 48 h the differences between Equi-Pro™ and the other extenders were even more pronounced. The viability of the semen was also lower in the Equi-Pro™ samples when compared to all other extenders. According to the manufacturer of Equi-Pro™ (Minitüb, Tiefenbach, Germany) this extender is a modification of the proven Kenney skim milk-glucose formulation [5] and was found to be suitable for storage of cooled stallion semen [13-15]. The Equi-Pro™ extender used in this study came from various batches and was stored and prepared according to the manufacturer's instructions and showed a similar effect on all stallions. There is no obvious explanation why in Icelandic stallions results with Equi-Pro™ were significantly poorer compared to the other extenders tested.

Besides the composition of the extender, the amount of seminal plasma may also influence the quality of cold-stored semen. It is known that both large or small amounts of seminal plasma can impair the motility of chilled semen, thus a concentration of 5-20% is generally recommended [16]. Ejaculates of Icelandic stallions were diluted to a final concentration of 30 x 10^6 sperm /mL. Depending on the sperm concentration in the ejaculate, the level of seminal plasma in the extended semen without centrifugation ranged from 6 to 50%. Differences in motility between centrifuged and not centrifuged semen can therefore be explained by the presence of variable amounts of seminal plasma. Furthermore it has been known that a high level of seminal plasma not only impairs sperm motility but may also affect DNA integrity of sperm [17].

Centrifugation of semen extended with INRA 82-egg yolk, INRA 96™ and GENT™ had a positive effect on motility but was detrimental when using Equi-Pro™. The interaction of extender and centrifugation has been reported in previous studies [17,18] which demonstrate that centrifugation of semen extended with Kenney and a modified (Tyrode-medium) Kenney extender had both negative and positive effects on the motility of cooled semen. In this context, it has been speculated that by adding an extender a beneficial effect is observed through the reduction in concentration of harmful compounds, but that this effect is countered by in the electrolyte balance. This phenomenon is particularly hard to explain when using extenders composed of natural materials such as skim milk,

the content of which is not clearly defined [18]. For optimal protection of cold-stored sperm it is therefore recommended to remove the majority of seminal plasma after centrifugation and to resuspend the sperm pellet with a chemically well defined extender [17].

In this study the 2 extenders INRA 82-egg yolk and GENT™, both containing egg yolk, showed similarly good preservation properties and after 48 h storage the quality of semen was slightly better with than without centrifugation. It has been known that extenders containing large amounts of egg yolk have a beneficial effect on sperm motility of cold-stored semen both with [16,19] and without [20] removal of seminal plasma. However, the combination of high amounts of egg yolk and seminal plasma may impair sperm motility and therefore when extenders containing more than 2% egg yolk are used, centrifugation should be performed prior to preservation [19]. In the present study the egg yolk content of INRA 82-egg yolk was 2%, whilst the precise formulation of GENT™ has not been declared by the manufacturer. Despite the relatively low egg-yolk concentration of INRA 82-egg yolk, centrifugation had a mildly beneficial effect on the motility of stored semen. INRA 96™ was the only medium with a chemically well-defined composition. It contains Hank's salts, glucose, lactose as well as native phosphocaseinate [4] and was developed for semen preservation at 15 °C under aerobic conditions [4,21]. In this study, the use of the INRA 96™ extender proved its worth for semen storage in anaerobic conditions at 5 °C, as the centrifuged INRA 96™ aliquots had the highest sperm motility after 48 h storage. Without centrifugation, however, the motility values were significantly lower than those diluted with INRA 96™ which had the seminal plasma removed by centrifugation. Regardless of this observation Le Frapper et al. [14] could demonstrate that progressive motility in not centrifuged semen extended with INRA 96™ was superior to that of semen processed with EquiPro®, EquiPro® Cell Guard™, Kenney's as well as *E-Z* Mixin™ after cooled storage for 24 and 48 h. Also when removing most of the seminal plasma by centrifugation, motility of cooled sperm stored for 48 h and 72 h was better maintained when using INRA 96™ compared to other extenders as VMD-Z™ (V.M.D., Adrenok, Belgium) and Kenney [22]. Moreover, Batellier et al. [23] found a better sperm motility and higher per cycle fertility after cold storage of semen for 24 h with INRA 96™ than with the skim-milk based extender *E-Z* Mixin™. The good results obtained with INRA 96™ may be

explained by the special formulation of this extender. The structural aspects of native micellar caseinates contained in INRA 96™ associated with the high concentration of sugars may create a physical protection for the sperm cell membranes during preservation [24]. Moreover the antioxidative properties of native phosphocaseinate are thought to protect sperm membranes from oxidative stress caused by lipid peroxidation [23].

In conclusion this study demonstrates that the quality of raw semen in the Icelandic horse is comparable to that of other breeds and that chilled semen preservation is possible. With the exception of Equi-Pro™, all other tested extenders, INRA 82-egg yolk, INRA 96™, GENT™, were well-suited for cold-semen storage and the removal of seminal plasma by centrifugation was advantageous.

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